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Forkhead box protein P1 as a downstream target of transforming growth factor-β induces collagen synthesis and correlates with a more stable plaque phenotype

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ABSTRACT

Objective: Atherosclerosis is an inflammatory disease, modulated by plaque stabilizing and de-stabilizing cell populations such as infiltrating monocytes and vascular smooth muscle cells (vSMCs). Transcription factors regulating proliferation and differentiation of atherosclerosis relevant cell types are of interest in this context. The forkhead box transcription factor FoxP1 modulates monocyte differentiation. We studied FoxP1 expression in atherosclerotic tissue, correlated FoxP1 expression with plaque characteristics and identified associations between FoxP1 and plaque proteins.
Methods: 116 Atherosclerotic plaques from carotid endarterectomy samples were histologically classified (fibrous, fibroatheromatous, atheromatous) and subjected to semi-quantitative protein analysis. Macrophage, SMC content and intraplaque thrombus amount were determined histologically. FoxP1 expression was investigated by western blotting and immunohistochemistry. In addition FoxP1 was overexpressed *in vitro* to identify causal relations between FoxP1 and plaque proteins.
Results: FoxP1 expression was observed in SMCs, macrophages, endothelial cells and T-cells within the plaque. High SMC and collagen content correlated with increased FoxP1 isoform (72 kD and 95 kD) levels. 72 kD FoxP1 expression was lower in plaques containing intraplaque thrombus. FoxP1 correlated with active intraplaque TGFβ signaling. *In vitro* stimulation of SMCs with TGFβ resulted in increased FoxP1 levels. 72 kD FoxP1 correlated with expression of pro-fibrotic EGR-1 and increased Col1A1 expression.
Conclusion: FoxP1 is expressed by different cell types in atherosclerotic lesions and associated with more stable plaque characteristics and intraplaque TGFβ signaling. FoxP1 expression *in vitro* is induced by TGFβ, resulting in increased collagen and EGR-1 expression, providing a mechanism for the observed association with a more stable plaque phenotype.

1. Introduction

Atherosclerosis is uniformly conceived as an inflammatory disease of the arteries [1,2]. Unstable atherosclerotic lesions are characterized by lower numbers of smooth muscle cells and a reduced fibrous cap covering the lesion, Moreover, these lesions contain increased numbers of inflammatory cells [2]. The infiltration of circulating monocytes into the vascular wall and their differentiation to plaque macrophages is a critical component in the initiation and progression of atherosclerotic lesion development [3]. Transcription factors regulating proliferation and differentiation of atherosclerosis relevant cell populations are of interest in influencing atherosclerotic plaque stability towards a more stable or unstable phenotype. Recently, the forkhead box transcription factor FoxP1 was described as a key modulator of macrophage differentiation [4] and we now hypothesized that FoxP1 could be an important transcriptional regulator of atherogenesis.

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FoxP1 was first identified by Shu et al. as a glutamine rich factor that is expressed in a wide variety of adult and fetal tissues cell types and belongs to the FoxP subfamily of the fox-transcription factors [5]. Misregulation of FoxP1 is seen in several malignant neoplasms, including prostate cancer and B-cell lymphomas [6]. With regards to macrophage biology, FoxP1 has been shown to impair the transition of monocytes towards macrophages and acts as a repressor of c-fms, which codes for the receptor of the differentiation stimulating growth factor M-CSF (Macrophage Colony Stimulating Factor). Inflammatory cells are a major component of atherosclerotic lesions and infiltration of monocytes, and T-lymphocytes can already be observed in early lesions and fatty streaks [3]. The maturation of infiltrating monocytes towards tissue macrophages and eventually lipid-containing foam cells is a critical step in plaque progression, and the signaling events that control differentiation and survival of plaque resident macrophages are largely unknown. Recently, FoxP1 was implicated in the process of macrophage maturation and was described to regulate monocyte differentiation in response to integrin-engagement [4].

Furthermore, FoxP1 has been shown to be involved in cardiac muscle cell proliferation and differentiation, in which differential effects of FoxP1 in early and late cardiac development were observed [7]. The role of FoxP1 in vascular smooth muscle proliferation has not been described so far, although this indicates that FoxP1 is a transcriptional mediator of proliferation and differentiation in different atherosclerosis relevant cell types [8]. As the process of muscle cell proliferation and differentiation is regulated by downstream effects of growth factors like TGF-β and FGF, we reasoned FoxP1 might be a downstream target of these growth factors.

FoxP1 might be further of influence on inflammatory plaque characteristics, by influencing the expression pro-inflammatory or anti-inflammatory proteins [4]. Besides c-fms, other downstream targets of FoxP1 however have been poorly described. Recently, a binding site of FoxP1 in the Interleukin-2 promoter region has been reported [9], but the exact effect of FoxP1 on interleukin expression is incompletely understood. Although some evidence is pointing towards a downregulatory effect of FoxP1 overexpression on specifically IL2, Bettelli et al. were not able to demonstrate an effect of FoxP1 overexpression on IL-2 expression [10]. The association of interleukins and plaque stability has been described in detail, showing opposing correlations of the different interleukins and plaque phenotype. For instance, IL-10 expression is higher in stable plaques whereas IL-8 expression is increased in unstable lesions [11]. Thus, FoxP1 might be relevant for the modulation of the intraplaque inflammatory balance by influencing the expression of different interleukins.

In this study we aimed to investigate the meaning of FoxP1 expression in atherosclerosis by examining the expression pattern of FoxP1 in atherosclerotic lesions and the correlation of its expression with the severity of atherosclerosis.

2. Materials and methods

2.1. Study design: the Athero-Express

The Athero-Express is a large multicenter patient study in which endarterectomy specimens are collected and characterized in combination with follow-up data. Medical ethical committees of all Dutch participating centers approved the study [12]. Each patient signed an informed consent form. Table 1 shows the baseline characteristics of the randomly selected patients for the current study.

2.2. Immunohistochemistry

The culprit lesion of 116 plaques was cut into 5 μm sections for histological analysis of plaque phenotype, the amount of intraplaque thrombus and collagen, smooth muscle cells, macrophages and fat content and classified as moderately, heavily or not stained as previously described [12]. As for the phenotype classification, plaques were divided into a fibrous, fibroatheromatous and an atheromatous group, as described previously [12]. The adjacent segment was used for protein isolation.

Table 1
Baseline characteristics.

Variable	(n = 126)
Age (yr)	64.5 ± 8.4
Sex (%)	
Female	29.9
Male	70.1
Diabetes (%)	16.0
High blood pressure (%)	69
Hyperscholesterolemia (%)	53.9
Smoking (%)	31.4
Statine use (%)	65.1
Beta-blocker use (%)	49.2
Anticoagulant use (%)	15.1
Calcium antagonist use (%)	23.0
ACE-inhibitor use (%)	41.3
Platelet aggregation inhibitor use (%)	95.2
Symptomic patients (%)	88.2

For histological evaluation of FoxP1 expression in different arterial wall layers of an atherosclerotic diseased vessel, after deparaffination and rehydration, endogenous peroxidase activity was blocked with methanol containing 0.3% peroxide. Heat-induced antigen retrieval was performed using 10 mM Tris-HCl and 1 mM EDTA, pH 9.0. Then sections were covered with serum-free protein block (Dako), followed by different primary antibodies. We stained the plaques for mouse anti-human FoxP1 (JC12, Abcam), rabbit anti-human CD3 (SP7; Labvision, Fremont, CA), mouse anti-human CD68 (PGM1, Dako) and mouse anti- alpha-smooth muscle cell-actin (1A4, Sigma–Aldrich). As a secondary agent we used Envision anti-rabbit (Dako) or Poly-AP anti-mouse (Immunovision Technologies, Brisbane, USA). As chromogens we used DAB and Liquid Permanent Red Kit (both from Dako).

2.3. FoxP1 protein expression levels

Of every patient we used 10 μg plaque protein, isolated as previously described, [12] which was reduced using 0.1 M DTT and 5 min of cooking. Proteins were run on a 10% polyacrylamide gel. Four μl of protein from cultured human monocytes (THP-1 cells) served as a positive control. After gels were run at 30 mA during 45 min, proteins were blotted on a Immobilon-FL transfer membrane (Millipore).

The membrane was blocked for 1 h at room temperature using 3% non-fat dry milk in PBS containing 0.1% Tween. Afterwards, the membranes were incubated overnight with a rabbit polyclonal FoxP1 antibody (0.15 μg/ml, ab16645, Abcam). A HRP-labeled goat-anti rabbit secondary antibody (P0447, Dako) was used at a concentration of 1 μg/ml. ß-Actin (clone AC-74, Sigma) expression levels were used to correct for the intraplaque cell number as previously described [13].

2.4. Influence of the TGF co-receptor endoglin and TGF on FoxP1 expression

We cultured Human aortic SMCs (HA-SMCs, CRL-1999, American Type Culture Collection) according to the manufacturer. We used an adenoviral endoglin RNAi construct at a multiplication of infection (MOI) of 250 to inhibit endoglin expression as described previously [13]. Adenoviral GFP was used as a control. Cells were washed after 16 h and allowed to recover for 24 h prior to starva-

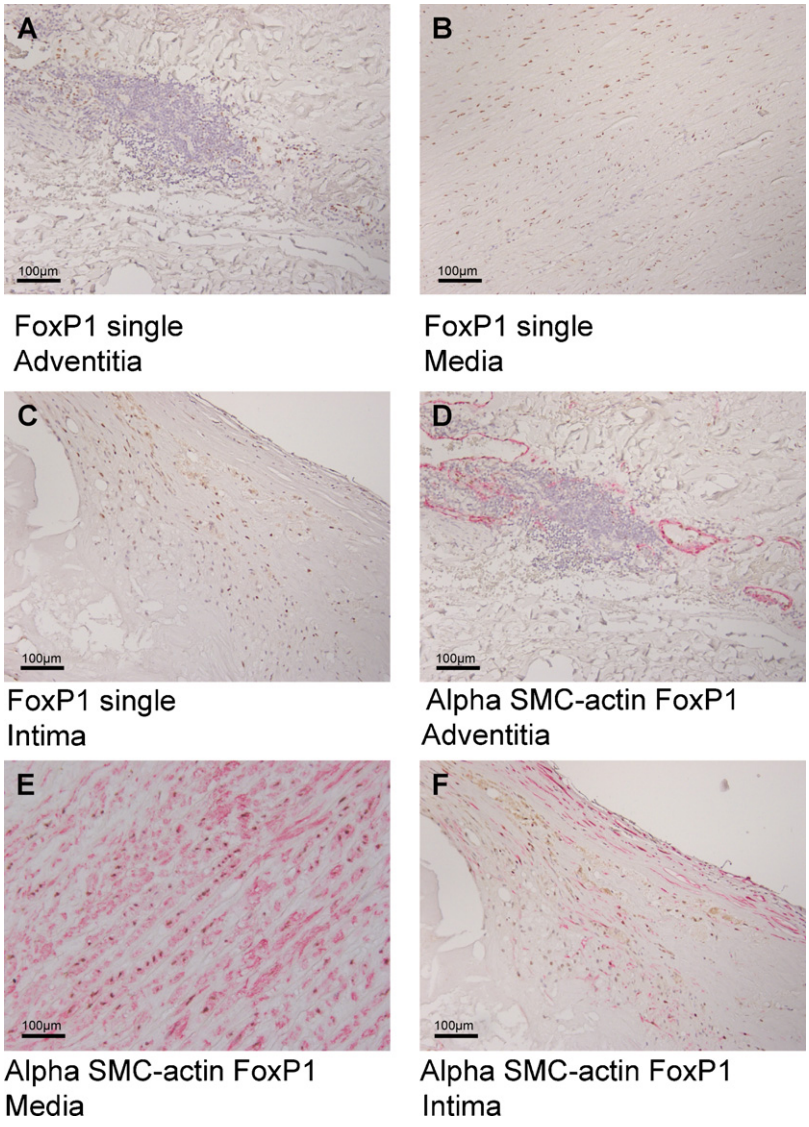

Fig. 1. FoxP1 positive cells in different layers of an atherosclerotic artery (A–C). Expression was especially high in the media (1B). FoxP1 positive SMCs (D–F) were observed in all layers of the vessel wall. The majority of endothelial cells was also FoxP1 positive (G). We observed FoxP1 staining in macrophage (H and I) and to a much lesser extent in T-cells (J and K). Nuclear staining in blue, FoxP1 nuclear staining in brown; CD34, CD68 and alpha smooth muscle cell positive staining in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

tion overnight before we stimulated with TGFβ1 (1 ng/ml) for 0, 1 h or 24 h. After washing twice with PBS, we isolated total cell protein using Tripure Isolation Reagent (Roche). Furthermore, we analyzed the effect of inhibiting TGFβ signaling using the ALK5 kinase inhibitor on FoxP1 expression. For this the ALK5 kinase inhibitor (SB-431542) was added to the cell culture in a final concentration of 1 μM in combination or in absence of 24 h of TGFβ stimulation (0.5 ng/ml) as described previously [14]. FoxP1 expression was analyzed using western blots as described above.

2.5. Influence of FoxP1 knock-down on TGFβ induced vascular smooth muscle cell proliferation

VSMC were seeded in a 96-well ELISA plate in 100 μl 10% SMC medium in 2 different concentrations: 0.5×10^4 and 1×10^4 cells per well. The next day the cells were transfected, using lipofectamine rnaimax, with siRNA foxp1 #2 and #6 as well as an untransfected control condition. After 5 h, the media was changed to 100 μl of 10% SMC with heat-inactivated FBS and incubated overnight at 37 °C. In the morning the media was changed to 100 μl of 10% SMC with heat-inactivated FBS plus 10 μl BrdU-labeling solution; also, plus or minus TGFβ (1 ng/μl). On day 4, the ELISA was performed according to the Roche protocol.

2.6. FoxP1 in vitro overexpression and quantitative RT-PCR of downstream targets

Transfection of HEK-293 cells with a FoxP1 plasmid was performed as described previously [13]. We seeded Human Embryonic Kidney (HEK) 293 cells (ATCC: CRL-1573.) in a 12 well plate and grown until 80% confluency in DMEM supplemented with 10% Fetal Calf Serum, 1% Penicillin/Streptomycin. We washed the cells twice with PBS and then added 0.4 ml of serum free DMEM. Using Lipofectamine™ Plus (Invitrogen), we transfected cells with 0.4 μg FoxP1 plasmid ($n = 4$) or an equal amount of GFP-vector as control ($n = 4$).

To provide insights into the consequences of increased expression of FoxP1 in an *in vitro* model, we analyzed which downstream targets are affected. We developed primers directed against components of the TGFβ signaling pathway. All expression levels

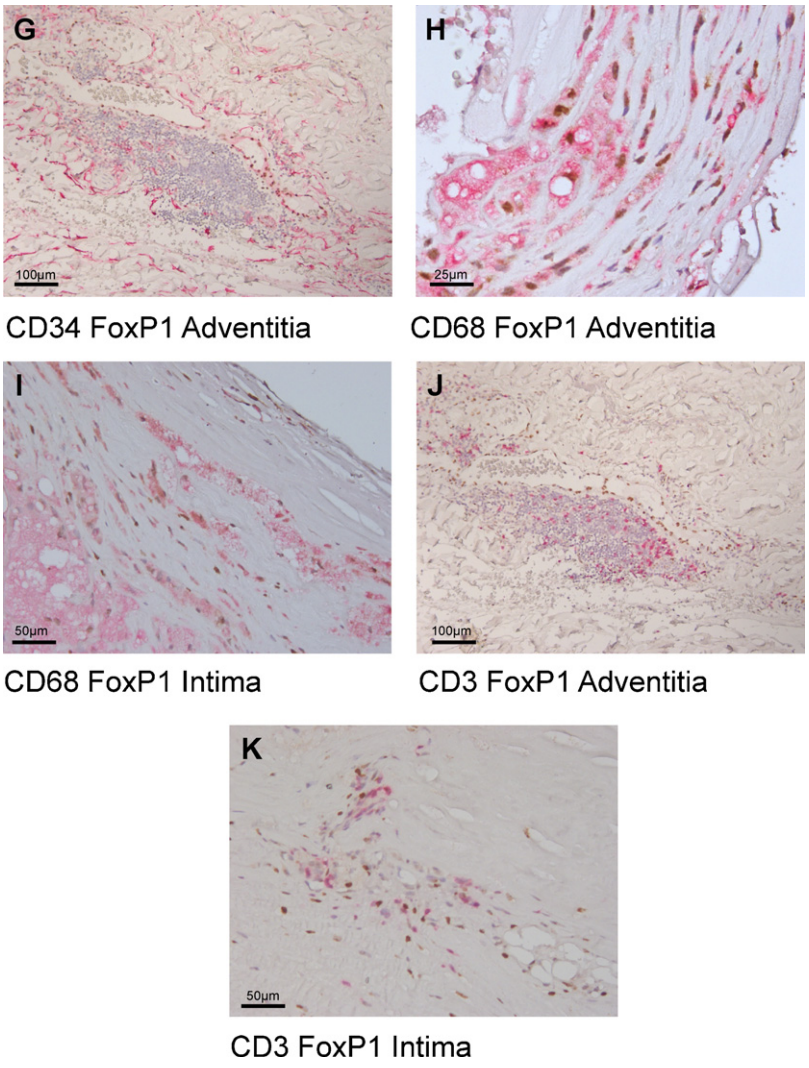

Fig. 1. (*Continued*)

were corrected for GAPDH expression. In Table 1 the different primer sequences are depicted. Functionality of the FoxP1 plasmid was tested by analyzing FoxP1 expression levels in FoxP1 transfected cells compared to GFP-transfected cells, using FoxP1 specific primers.

2.7. Statistical analysis

Data are presented as mean and 95% confidence interval. As our data was not-normally distributed, we used the non-parametric Mann–Whitney (for comparison of two categories) or Kruskal–Wallis tests (for comparison of 3 or more categories). Correlations were analyzed using the Spearman–Rank Test. For the statistical analysis of the *in vitro* experiments we used a Mann–Whitney test. *P*-Values of <0.05 were considered significant.

3. Results

3.1. FoxP1 is expressed in atherosclerotic lesions by different cell types

As depicted in Fig. 1A–C, we observed FoxP1 expression in different vascular layers of atherosclerotic vessel. FoxP1 staining was mainly restricted to the nucleus of cells in the intima, the media and adventitia, with most pronounced staining in the media.

Using double staining techniques, we indeed observed intranuclear staining of FoxP1 in smooth muscle cells in the different vascular layers, with most of these cells expressing FoxP1 (Fig. 1D–F). Using double staining for CD34 and FoxP1 we also observed co-localization in the majority of endothelial cells (Fig. 1G), which might indicate a stabilizing effect of FoxP1 on vascular integrity, as knock-down of FoxP1 has been described to result in severe perivascular hemorrhage [15]. Furthermore, although expression levels of FoxP1 have been shown to decrease after the transition of monocytes to macrophages [4], we still observed FoxP1 expression in macrophages as these cells were double stained for FoxP1 and CD68 (Fig. 1H and I). Using CD3 staining, we analyzed if FoxP1 is expressed by T-cells, but only a minority of T-cells in the atherosclerotic vessel express FoxP1 (Fig. 1J and K).

3.2. FoxP1 expression is higher in fibrous plaques

In a second step we analyzed if FoxP1 expression would differ in plaques with a fibrous phenotype compared to an atheromatous phenotype, generally regarded as more unstable [2,16]. Using western blots, we observed two separate bands of FoxP1-expression, a 95 kD isoform as well as a 72 kD isoform, which showed a significant correlation in expression levels ($p < 0.001$, correlation coefficient .609). For the 95 kD FoxP1 isoform, expression was significantly higher in plaques with a fibrous phenotype, compared to the more

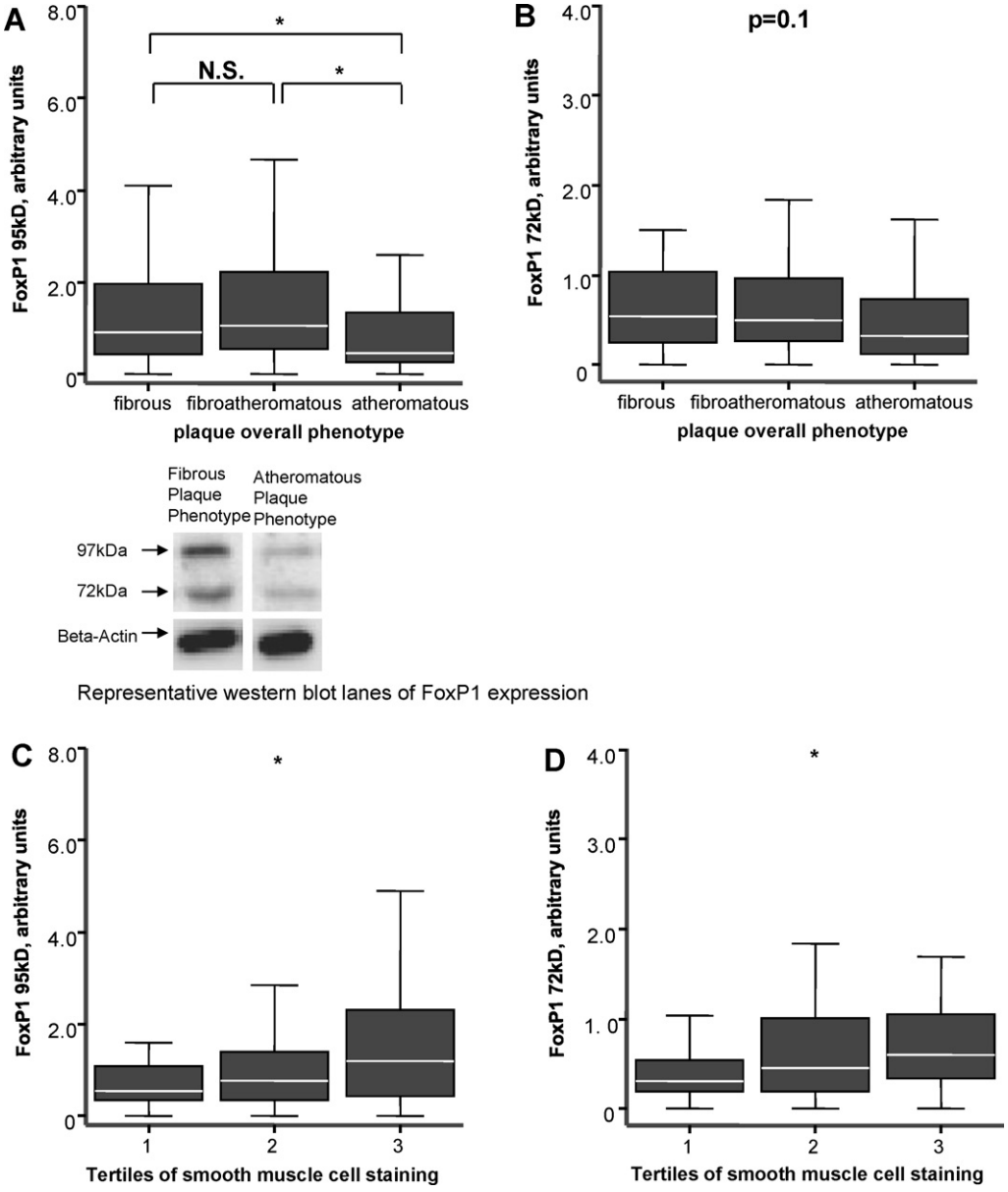

Fig. 2. FoxP1 expression correlated to histological plaque characteristics. Plaques with a fibrous phenotype showed increased expression of the 95 kD isoform of FoxP1, compared to atheromatous plaques (A). Definition of different plaque phenotypes was previously described [12]. For the 72 kD FoxP1 isoform we observed an non-significant trend towards a positive correlation with a more fibrous plaque phenotype (B). Both isoforms of FoxP1 were significantly correlated to the amount SMCs in the plaque. No significant correlation was observed between FoxP1 expression and the macrophage content of the plaque (C–E). The amount of intraplaque thrombus, which is associated with unstable plaques, showed a negative correlation with FoxP1 expression (F and G). * = $p < 0.05$.

atheromatous plaques ($p = 0.049$). The expression of the 72 kD isoform showed a non-significant increased expression in fibrous plaques compared to atheromatous plaques (p = 0.166). (Fig. 2A and B).

Plaques containing a high smooth muscle cell content revealed significantly higher expression levels of both isoforms (for FoxP1, 72 kD: $p = 0.027$, correlation coefficient = .211, FoxP1, 95 kD: $p = 0.026$, correlation coefficient = .201) (Fig. 2C and D). Neither the 95 kD nor the72 kD isoform of FoxP1 was significantly correlated to intraplaque macrophage content ($p = 0.199$ resp. 0.278) (Fig. 2E and F).

A factor with a strong association with plaque instability, the amount of intraplaque thrombus [17,18], was negatively correlated with the 72 kD isoform of FoxP1 ($p = 0.002$). For the 95 kD variant, we observed a trend towards a negative correlation ($p = 0.106$) (Fig. 2G and H).

3.3. FoxP1 and TGFβ signaling

Both the FoxP1 isoform of 95 kD as well as the 72 kD isoform correlated to the phosphorylated TGFβ transcription factor pSMAD2 (with respectively a correlation coefficient: 0.500, $p < 0.01$, and correlation coefficient: 0.609, $p < 0.01$), indicating that FoxP1 might be a downstream target of TGFβ or that FoxP1 stimulates TGFβ expression (Fig. 3A and B). We did not find evidence for a stimulating effect of FoxP1 overexpression on TGFβ expression levels, whereas, *in vitro* stimulation of vSCMs with TGFβ led to an upregulation of both FoxP1 isoforms (Fig. 3C and F). Additionally, knock-down of FoxP1 using siRNA resulted in significantly decreased TGFβ induced vascular smooth muscle cell proliferation (Fig. 3I).

To further analyze the role of FoxP1 in TGFβ signaling we analyzed the correlation of FoxP1 expression in the plaque and the expression of the TGFβ co-receptor endoglin [19–21]. The 72 kD

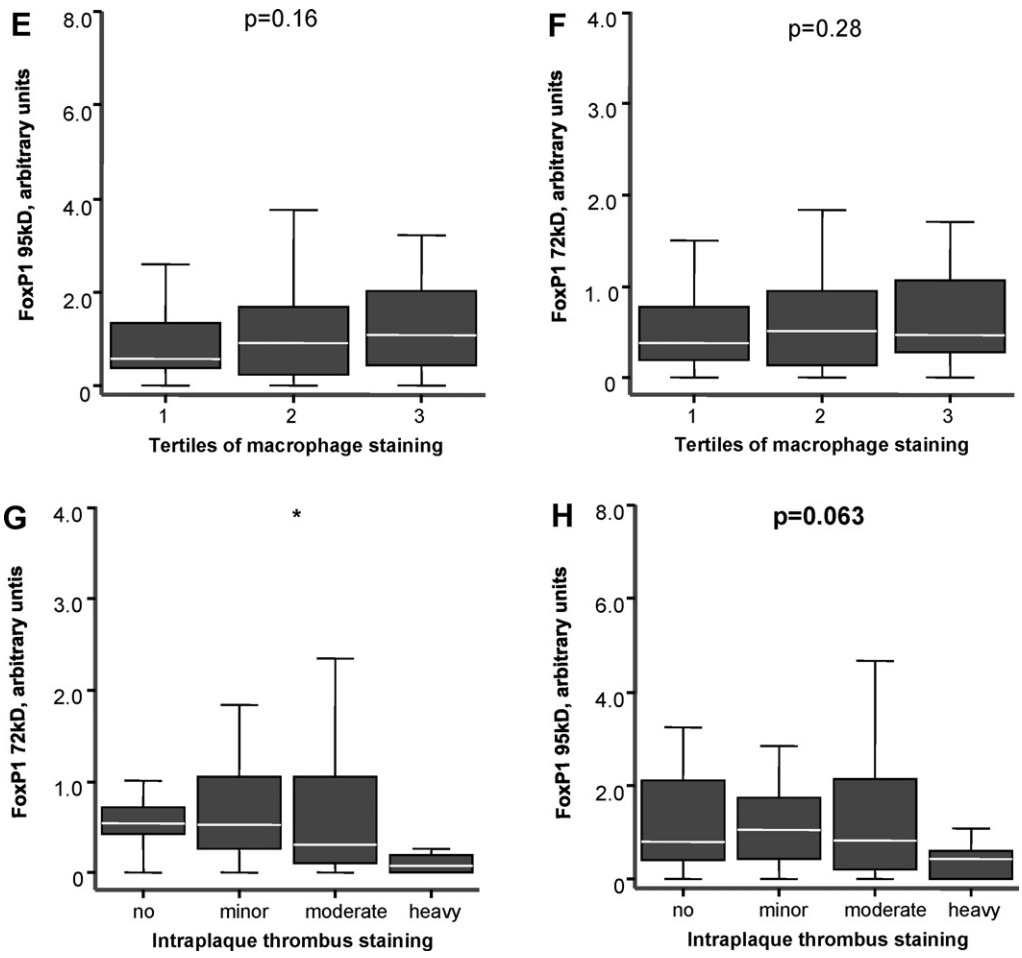

Fig. 2. (*Continued*)

isoform of FoxP1 correlated positively to the TGFβ co-receptor endoglin ($p < 0.05$), which could be due to a stimulatory effect of FoxP1 on endoglin expression or a downstream effect of endoglin expression on FoxP1 signaling (Fig. 3D and E). Interestingly, in the absence of endoglin using RNAi, TGFβ failed to induce FoxP1 expression. *In vitro* overexpression of FoxP1 resulted in a 3.3-fold increase in endoglin mRNA levels ($p < 0.05$), suggesting a primarily modulatory effect of FoxP1 on TGFβ signaling (3G). Inhibition of the ALK5 receptor led to the absence of a stimulatory effect of TGF on FoxP1 95 kD expression, while no significant effect was observed on FoxP1 72 kD expression (Fig. 3H).

3.4. FoxP1 expression correlates to IL2, Il-4 and IL-10

Interestingly, the expression level in the plaques of the 95 kD band of FoxP1 correlated positively with three different interleukins, both the anti-inflammatory interleukins IL-4 ($p = 0.034$, correlation coefficient: .202) and IL10 ($p = 0.026$, correlation coefficient: .212) as well as the pro-inflammatory IL-2 ($p = 0.037$, correlation coefficient: .199, $n = 110$) (4A-C). As FoxP1 is known as a transcriptional repressor [5], these positive associations might be indirect and caused by inhibition of another repressing factor. *In vitro*, we analyzed the causal effect of an increase in FoxP1 levels, which led to a 5-fold increase in IL-2 levels ($p < 0.05$) and a 10.2-fold increase in IL-4 ($p < 0.05$) (Fig. 4D and E). IL-10 levels were below detection limit.

3.5. FoxP1 and smooth muscle cell and collagen content

Based on our finding that the expression levels of both FoxP1 isoforms are significantly higher in SMC rich lesions, we analyzed the potential mechanism for this observation. FoxP1 72 kD correlated significantly to the transcription factor EGR-1 (correlation coefficient = 0.292, $p = 0.041$), which has been shown to stimulate smooth muscle cell proliferation as well as collagen synthesis (Fig. 4F) [13,22,23]. The 95 kD FoxP1 isoform was not significantly correlated to EGR-1 expression. *In vitro*, we demonstrated that increased FoxP1 levels resulted in a significantly 2.5 fold increased EGR-1 expression ($p < 0.05$), suggesting that FoxP1 might increase smooth muscle proliferation by upregulation of EGR-1 (Fig. 4G). As smooth muscle cells are an important source of collagen production, and a high percentage of the smooth muscle cells stained positive for FoxP1, we analyzed if FoxP1 affected collagen production. We observed a 1.7-fold increase significant increase in Col1A1 mRNA levels after FoxP1 overexpression ($p = 0.011$) (Fig. 4H).

4. Discussion

To the best of our knowledge, this is the first study to demonstrate that FoxP1 is a transcription factor downstream of TGFβ, which is expressed by different cell types in atherosclerotic lesions. In atherosclerotic plaques FoxP1 was mainly expressed by smooth muscle cells and endothelial cells, although we also observed sub-

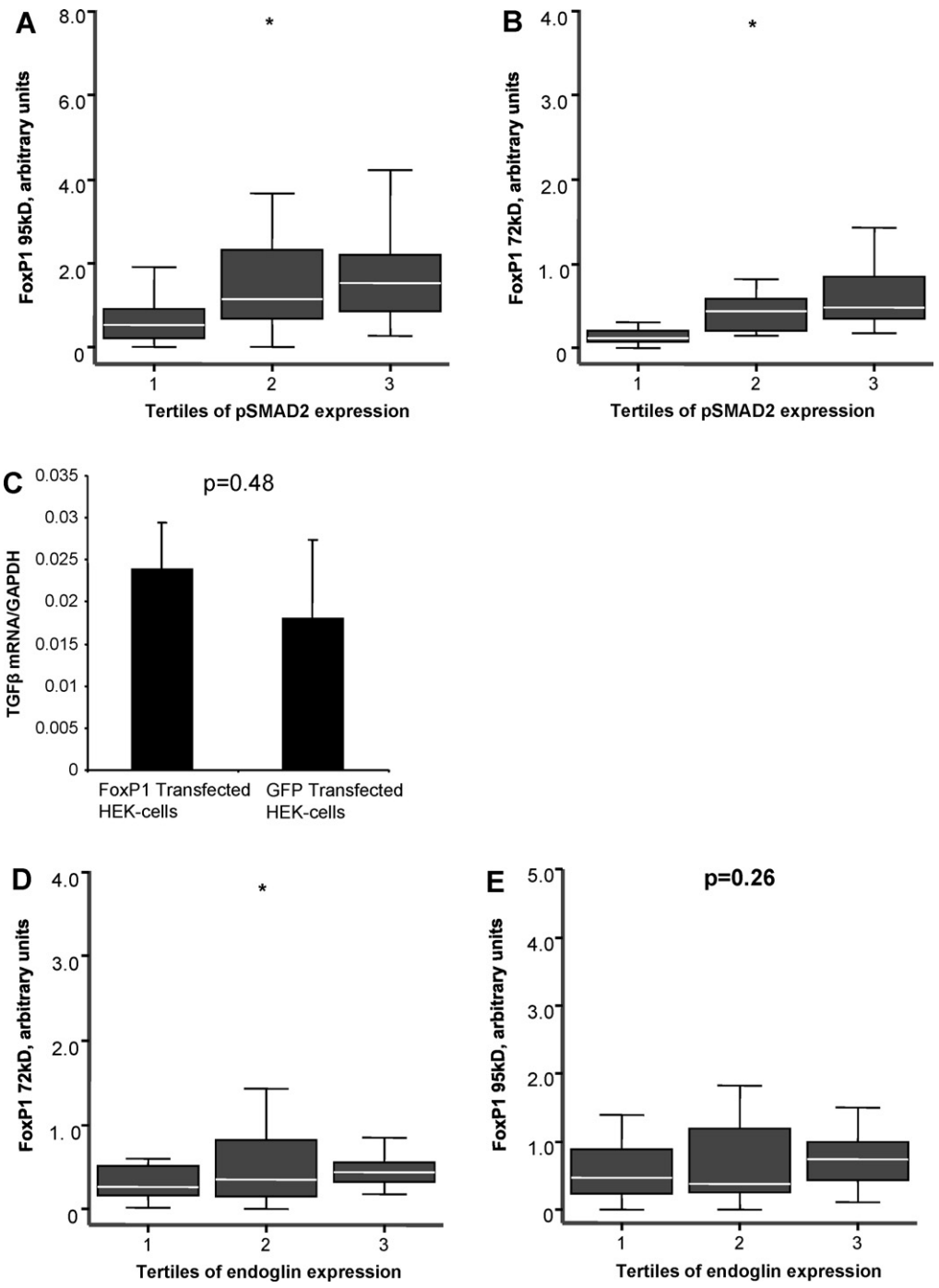

Fig. 3. Association of FoxP1 expression and the TGFβ signaling pathway. Both isoforms of FoxP1 correlated significantly with the activated form of the downstream transcription factor of TGFβ, phosphorylated SMAD-2 (A and B) suggesting the involvement of FoxP1 in the TGF-beta signaling pathway. This was not mediated by an increase in TGFβ-levels by FoxP1 as *in vitro* overexpression of FoxP1 did not lead to an increase in TGFβ mRNA. (C). A positive correlation between the 72 kD FoxP1 isoform and the TGFβ co-receptor endoglin was observed, suggesting a modulating effect of TGFβ signaling via its co-receptor, endoglin (D and E). Indeed, RNA inhibition of endoglin resulted in the inability of TGFβ to induce FoxP1 expression (mainly the 72 kD variant) (F). Additionally, *in vitro* overexpression of FoxP1 resulted in increased mRNA expression of endoglin, suggesting a positive feed back mechanism (G). Vascular smooth muscle cells failed to increase FoxP1 95 kD expression after TGFβ stimulation in the presence of an ALK5 kinase-inhibitor, strengthening the idea of a crucial role for endoglin in TGFβ induced FoxP1 expression (H) Knock-down of FoxP1 using siRNA resulted in decreased TGFβ induced stimulation of vascular of smooth muscle cell proliferation (I). * = $p < 0.05$. Non-categorical data are divided into equal tertiles for graphical representation.

stantial staining by macrophages and to some extent by T-cells. As the staining pattern of this transcription factor was mainly nuclear and not cytoplasmatic, FoxP1 most likely also signals actively in the atherosclerotic plaque. FoxP1 expression correlated to active intraplaque TGFβ signaling as the levels of the activated form of the transcription factor downstream of TGFβ, pSMAD2 [24] showed a highly significant correlation to FoxP1. *In vitro*, using cultured vascular smooth muscle cells, we were able to increase FoxP1 levels after addition of TGFβ, indicating a stimulatory downstream effect of this growth factor on FoxP1. Several lines of evidence suggest a plaque stabilizing role for TGFβ, although the mechanism by which TGFβ promotes plaque stability are presently unknown [13,22,25]. We suggest that FoxP1 is in part responsible for these effects. A limitation of this study is the relatively low correlation

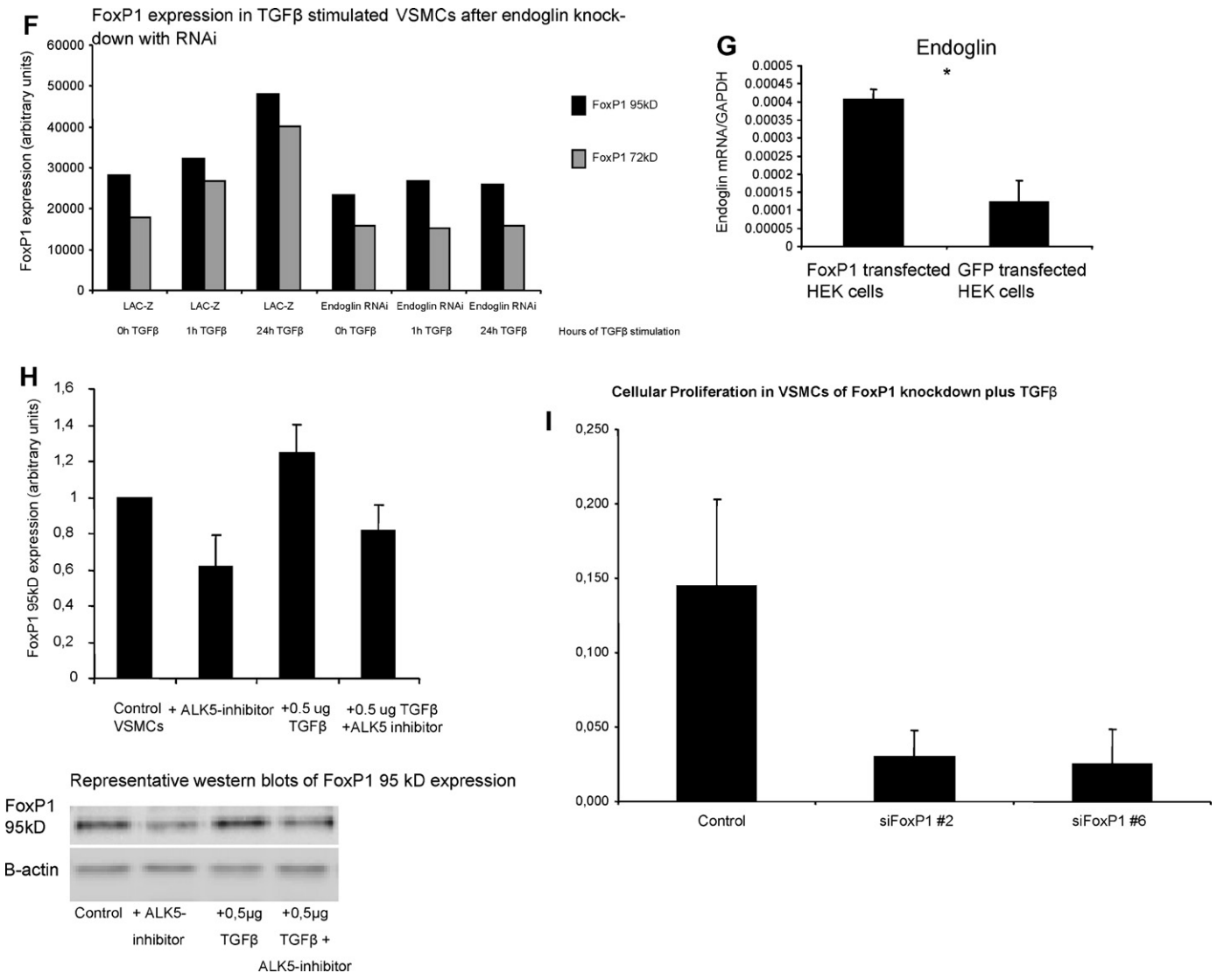

Fig. 3. (*Continued*)

coefficients which are consistent with a heterogeneous disease process as atherosclerosis is.

In our carotid artery specimens we detected two isoforms of FoxP1, a 95 kD and a 72 kD variant. Previous studies suggest that the FoxP1 protein diversity is increased due to alternative splicing [26]. The 95 kD variant showed a significantly increased expression in fibrous plaques, compared to atheromatous and fibroatheromatous phenotypes *In vitro* we were able to demonstrate a stimulatory effect of FoxP1 overexpression on collagen-synthesis as the expression of Col1A1 mRNA levels increased after overexpression of FoxP1, further implying a stabilizing effect of FoxP1 on atherosclerotic plaques. Recently, in the fibrotic pathological process of heart failure, FoxP1 expression was shown to be increased [27], which supports a fibrosis stimulating role of FoxP1. A significant positive correlation between the FoxP1 isoform of 72 kD and the intraplaque levels of the pro-fibrotic transcription factor EGR-1 provides additional evidence for a potential stabilizing role of FoxP1 in atherosclerosis [13,22].

A highly significant correlation of both FoxP1 isoforms and the activated form of the intracellular effector of TGFβ, pSMAD2 put forward the involvement of FoxP1 in the TGFβ signaling cascade. This could either imply that FoxP1 stimulates transcription of TGFβ, leading to increased phosphorylation of pSMAD2, or that FoxP1 is a downstream effector of TGFβ. We did, however, find evidence for the second suggestion only, as FoxP1 overexpression did not lead to increased TGFβ mRNA levels. The TGF co-receptor endoglin, known to modulate the downstream signal of TGFβ [20,21] also significantly correlated to FoxP1 72 kD expression levels. We did observe a significant increased expression of both endoglin and EGR-1 levels after FoxP1 overexpression, which is in line with the observed associations in our patient material. These findings might indicate that the upregulation of EGR-1 after FoxP1 overexpression was not mediated via an increase in TGFβ levels, but more likely by a modulatory effect on TGFβ signaling by upregulation of the TGFβ co-receptor endoglin. Inhibition of endoglin expression using RNAi, abolished the stimulatory effect of TGFβ on FoxP1 expression, which was especially the case for the 72 kD isoform. This implies that besides a stimulatory effect of FoxP1 on endoglin expression, the expression of endoglin is obligatory for the stimulatory effect of TGFβ on FoxP1-expression. Furthermore, using a downstream blocker of TGFβ signaling, the ALK5 kinase-inhibitor SB-431542, we observed decreased levels of FoxP1 after TGFβ stimulation compared to control cells that were only stimulated with TGFβ. Although FoxP1 has been described mainly

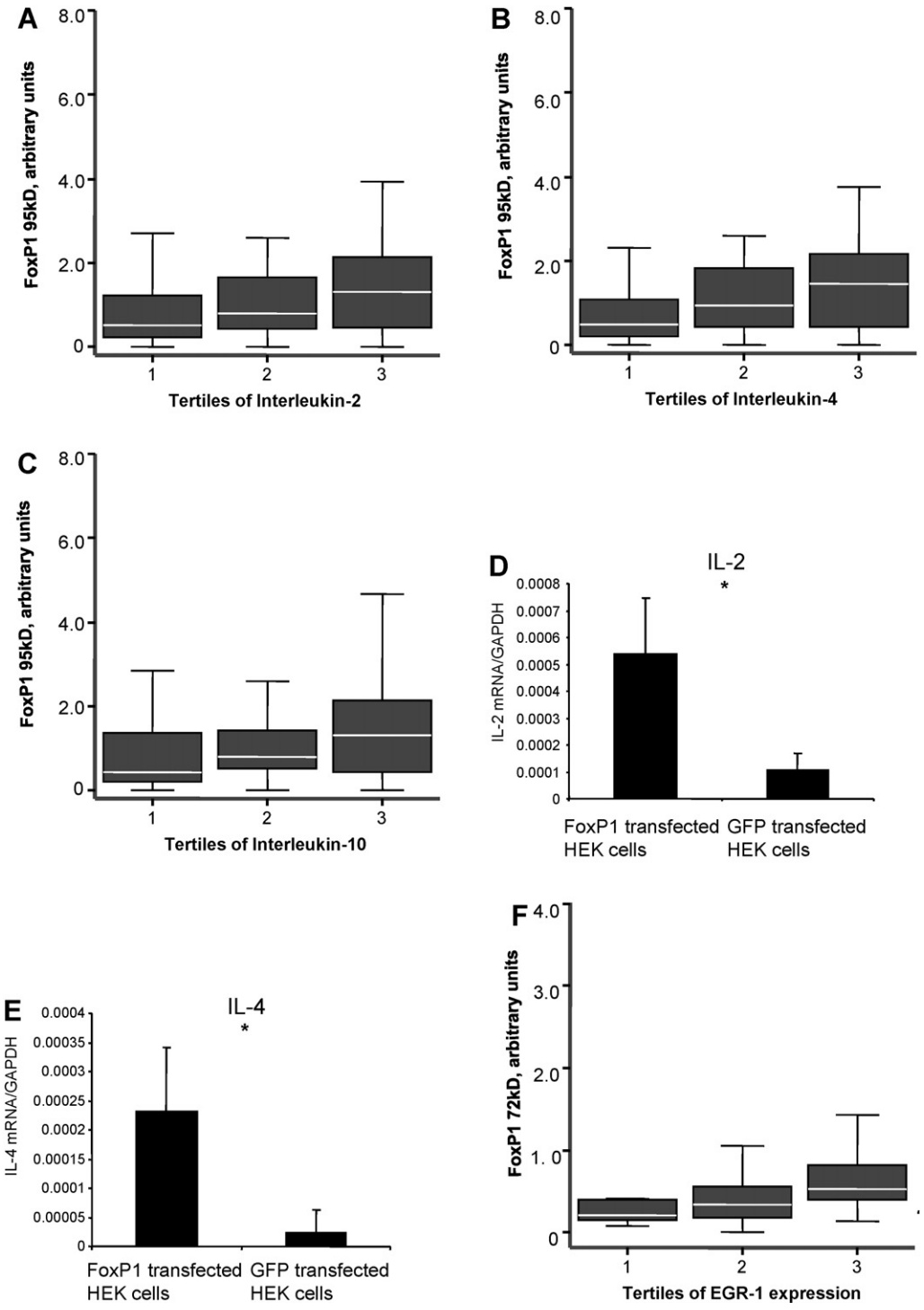

Fig. 4. FoxP1 association with plaque stability mediating proteins. Downstream effects of FoxP1. FoxP1 95 kD correlated with interleukin-2, 4 and 10 (A–C) and *in vitro* overexpression of FoxP1 resulted in increased IL-2 and IL-4 mRNA levels (D and E). FoxP1 (72 kD variant) expression was also associated with the expression of the TGFβ transcription factor EGR-1, which is involved in smooth muscle proliferation. (F). Overexpression of FoxP1 also resulted in increased EGR-1 levels (G). FoxP1 overexpression resulted in increased Col1A1 mRNA levels, suggesting a fibrotic effect of FoxP1 (H) $^{*} = p < 0.05$. Non-categorical data are divided into equal tertiles for graphical representation.

as a transcriptional repressor [6,28], potentially it also possesses the capacity to stimulate the expression of several downstream targets. On the other hand, another possible explanation for our findings would be that FoxP1 represses an unknown target which contains inhibitory effects on endoglin or EGR-1 expression levels. This remains to be elucidated. Potential other downstream or upstream regulatory mechanisms of FoxP1 expression include effects of FoxP1 on NF-kappaB expression, although a causal interaction of these transcription factors has not been identified so far. Of interest is the recent observation that overexpression of FoxP3 results in decreased NF-kappaB expression [29]. The FoxP3 forkhead domain shares high sequence similarity with two other FoxP family members FoxP1 and FoxP2, and co-operates with FoxP1 and FoxP2 by means of heterodimerization. Based on these findings one can speculate on a potential interaction between FoxP1 and NF-kappaB, thereby modulating plaque stability characteristics.

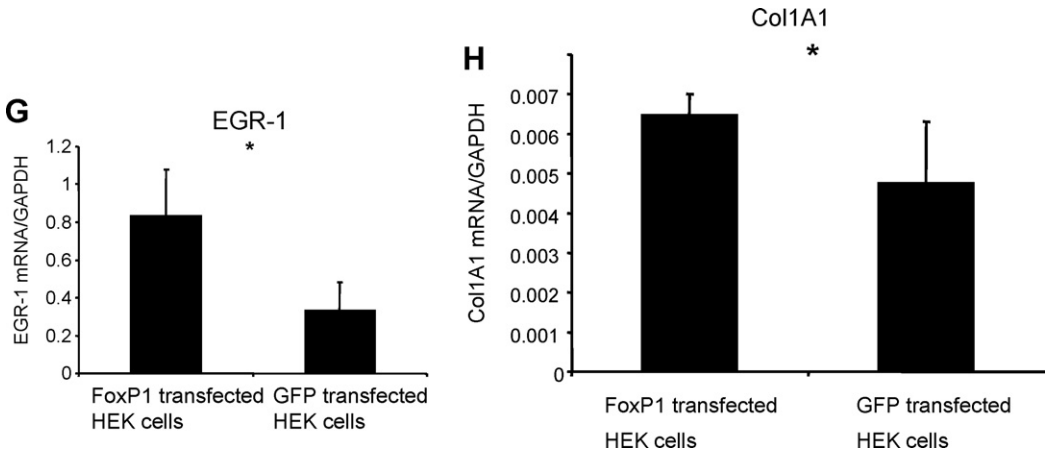

Fig. 4. (*Continued*)

As TGFβ and EGR-1 have been demonstrated to increase smooth muscle cell proliferation [23,24], we analyzed the correlation of FoxP1 and intraplaque smooth muscle cell content. We observed a positive association of both FoxP1 isoforms and a high smooth muscle cell content. This association could be due to the involvement of FoxP1 in smooth muscle cell proliferation, partly mediated by its effect on EGR-1 expression. These data indicate that FoxP1 could stimulate intraplaque smooth muscle cell content by increasing smooth muscle cell proliferation by upregulation of EGR-1. Knock-down of FoxP1 diminished the capacity of TGFβ to induce vascular smooth muscle cell proliferation, further demonstrating the involvement of FoxP1 in vascular smooth muscle cell proliferation.

The 72 kD FoxP1 variant was negatively correlated to the amount of intraplaque thrombus. Also knock-down of FoxP1 has been shown to result in severe perivascular hemorrhage, suggesting the involvement of FoxP1 in maintaining vascular integrity [15]. This finding might be of interest in the context of atherosclerosis as an import feature of unstable plaques is intraplaque hemorrhage, which can be caused by vascular leakiness [17,18].

Additionally, the 95 kD isoform of FoxP1 correlated positively to three different interleukins, IL-2, IL4 and IL10. Although significant, we observed a high variation necessitating future studies for the analysis of the biological relevance of these observations. IL4 and IL10 are associated with plaque stability whereas the effect of IL-2 has been discussed controversially [11]. Our observed correlation between FoxP1 and IL-2, Il-4 and IL-10 was confirmed *in vitro*, as FoxP1 overexpression resulted in increased levels of IL-2 and IL-4 (IL-10 levels were below detection limit). This might indicate that FoxP1 also has immune modulatory effects in the plaque. Conversely, Wang et al. demonstrated an inhibitory effect of FoxP1 overexpression on IL-2 levels [9], whereas Bettelli et al., observed no effect on IL-2 and a non-significant increase in IL-4 levels [10]. This might indicate that the effects of FoxP1 on interleukin expression are cell type, context or dose dependent [30]. We did not, however, find a significant negative correlation between FoxP1 expression and macrophage content. Although FoxP1 has been demonstrated to inhibit the transformation from monocytes into macrophages [4], little is known about active FoxP1 signaling once these cells become fully matured. It might be that FoxP1 is still active as a transcription factor once these cells are transformed into active macrophages.

We conclude that the FoxP1 transcription factor is a transcription factor downstream of TGFβ and is associated with fibrous plaques and collagen synthesis *in vitro* and might therefore beneficially modulate plaque stability.

Disclosures

None.

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None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.05.017.

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